Polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp *silvaticum* in long term cultures from Crohn's disease and control tissues

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Abstract

Thirty one cultures were established in MG3 medium from the intestinal tissues of 29 patients, including 18 with Crohn's disease, five with ulcerative colitis, and six non-inflammatory bowel disease controls. All cultures grew either acid fast bacilli or uncharacterised spheroplasts. Pellets from these cultures were coded and assayed blind for *M paratuberculosis* and *M avium* subsp *silvaticum* using IS900- and IS902-PCR (polymerase chain reaction) assays, respectively. IS900 and IS902 are multicopy DNA insertion elements specific for these two organisms. Six Crohn's disease cultures and a single non-inflammatory bowel disease control were positive for *M paratuberculosis*. A further six cultures were positive for *M avium* subsp *silvaticum*, of which two each were from Crohn's disease, ulcerative colitis, and non-inflammatory bowel disease controls. The intensity of the IS900-PCR signals indicated very low numbers of *M paratuberculosis* organisms and bore no relation to visible spheroplastic or bacillary mycobacterial growth. The results suggest that *M paratuberculosis* isolated from man exists in a form which hardly replicates if at all when cultured in MG3 medium in vitro, and are consistent with the involvement of this known animal enteric pathogen in a proportion of chronic enteritis in man.

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Mycobacterium paratuberculosis is the causative agent of Johne's disease, a chronic enteritis in ruminants and in other animals.[1] There has been interest in the potential role of *M paratuberculosis* in the pathogenesis of Crohn's disease since Dalziel[2] first drew attention to the similarities between Johne's disease in cattle and granulomatous enteritis in man (Crohn's disease). In recent years, analysis of long term cultures of Crohn's disease and other human intestinal derived tissues by several laboratories have yielded a variety of bacillary and spheroplastic forms of mycobacteria.[3-5] The mycobacterial species detected were generally those species found in apparently healthy individuals.[6] *M paratuberculosis* has been cultured from Crohn's disease tissues but only after prolonged incubation and only in a very small minority of cases.[7-10] *M paratuberculosis* grows only very slowly cultured in vitro and some animal isolates have been especially difficult to culture, particularly those strains isolated from sheep.[11]

IS900 is a multicopy DNA insertion element, unique to the genome of *M paratuberculosis*.[12] Polymerase chain reaction (PCR) amplification, capable of both highly specific and sensitive detection of *M paratuberculosis* has been developed, based upon the DNA sequence of IS900.[13] IS900-PCR is capable of distinguishing *M paratuberculosis* from even the very closely related *Mycobacterium avium* subsp *silvaticum*. *M avium* subsp *silvaticum* is also known to cause chronic enteritis in animals[14] and contains IS902, a DNA insertion element unique to this mycobacterial species. IS902 shares homology with IS900.[15] In this paper we report the use of IS900- and IS902-based polymerase chain reaction for the specific detection of *M paratuberculosis* and *M avium* subsp *silvaticum* in long term cultures from Crohn's disease, ulcerative colitis, and non-inflammatory bowel disease tissues.

Methods

MYCOBACTERIAL CULTURES

Long term in vitro cultures were established in MG3 broth from resection and endoscopic biopsy samples of intestinal tissue as described previously.[16] Tissue samples from 18 patients with Crohn's disease, five with ulcerative colitis, and six non-inflammatory bowel disease controls were cultured at 37°C for between two and six years and yielded acid fast bacilli or spheroplasts (Table). Also included in this study were known cultures of *M avium* serovar 2, *M paratuberculosis* strain (Linda), *M fortuitum* ATCC 6841, *Mycobacterium kansasii* ATCC 12478 and MG3 medium only. Cultures were pelleted by centrifugation at 17 000 *g* for 15 minutes, coded in Houston then sent to London to be assayed. Coded samples were assayed blind.

DNA EXTRACTION AND POLYMERASE CHAIN REACTION ASSAY

The coded long term culture pellets were resuspended in 100 µl of TE (10 mM Tris; 0·1 mM EDTA pH 8·8) then boiled for 20 minutes in 1·5 ml screw capped tubes to release microbial DNA. Tubes were centrifuged at 17 000 *g* for five minutes and the supernatants were recovered.

TABLE *Tissue samples*

Code	Tissue	Isolate	IS900 PCR	IS902 PCR
30	CD (B)	S	+	−
50	CD (B)	S	−	−
54	CD (B)	AFB	−	−
57	CD (B)	AFB	−	−
59	CD (B)	AFB	−	−
29	CD (R)	S	+	−
32	CD (R)	S	+	−
33	CD (R)	S	−	+
35	CD (R)	S	+	−
37	CD (R)	S	−	−
39	CD (R)	S	−	−
40	CD (R)	S	−	−
48	CD (R)	S	−	−
49	CD (R)	S	+	−
51	CD (R)	S	−	−
52	CD (R)	S	−	+
63	CD (R)	S	−	−
64	CD (R)	S	+	−
44*	UC (B)	AFB	−	+
60*	UC (B)	AFB	−	−
45#	UC (B)	AFB	−	−
58#	UC (B)	S	−	−
61	UC (B)	AFB	−	+
42	UC (R)	S	−	−
62	UC (R)	S	−	−
46	Non-IBD (B)	S	−	+
53	Non-IBD (B)	AFB	−	+
55	Non-IBD (B)	AFB	+	−
56	Non-IBD (B)	AFB	−	−
43	Non-IBD (R)	S	−	−
47	Non-IBD (R)	S	−	−
31	−	Medium only	−	−
34	−	*M avium* 2	−	−
36	−	*M paratuberculosis*	+	−
38	−	*M fortuitum*	−	−
41	−	*M kansasii*	−	−

CD=Crohn's disease; UC=ulcerative colitis; IBD=inflammatory bowel disease; R=resection, B=biopsy, S=spheroplast; AFB=acid fast bacilli;*=2 AFB from one tissue, #=an AFB and a spheroplast from one tissue.

POLYMERASE CHAIN REACTION PRIMERS AND CONDITIONS

Oligonucleotide primers P90 (5′-GTTCGGGGCCGTCGCTTAGG-3′) and P91 (5′-GAGGTCGATCGCCCACGTGA-3′) were selected to amplify a unique 400 base-pair (bp) fragment of the 5′ region of IS900 (nucleotides 22-421).[12] From the 5′ region of IS902, primers P102 (5′-CTGATTGAGATCTGACGC-3′) and P103 (5′-TTAGCAATCCGGCCGCCCT-3′) were selected to amplify a 252 bp fragment (nucleotides 132-383), unique to IS902.[15] All primer sequences were selected on the basis of minimal 3′ base mis-match, freedom from involvement in predicted secondary structure in IS900 and IS902, and specificity in relation to the known DNA sequences of other related IS elements.[17-19] The specificity of IS900 polymerase chain reaction for *M paratuberculosis* has already been shown.[13 20 21] To demonstrate the specificity of the IS902 polymerase chain reaction used for *M avium* subsp *silvaticum*, genomic DNA from 25 bacterial species including 21 mycobacteria (see legend for Figure 1) was subjected to IS902 polymerase chain reaction in accordance with the protocol described below.

For polymerase chain reaction assays, 5 μl supernatant sample was added to 45 μl reaction premix consisting of 67 mM Tris-HCl pH 8·8, 16·6 mM NH_4SO_4, 3·35 mM $MgCl_2$ (6 mM for IS902-PCR), 1·7 mg/ml BSA, 0·2 mM of each of dATP, dGTP, dCTP, dTTP, 300 ng each of primers P90 and P91 (P102 and P103 for IS902-PCR), 10 mM β-mercaptoethanol, 10 mM Tris; 0·1 mM EDTA and 2 units of Taq DNA polymerase (Amplitaq^{TM} Cetus). The reaction mixtures were overlaid with 50 μl paraffin oil, then submitted to 33 cycles of IS900-PCR amplification or 40 cycles of IS902-PCR amplification. Amplification conditions comprised of denaturation at 94°C for one minute, annealing at 58°C for one minute and primer extension at 72°C for three minutes. For each DNA extract, polymerase chain reaction assays were performed in triplicate. A negative polymerase chain reaction control (buffer only) and an appropriate positive polymerase chain reaction control (10 fg *M paratuberculosis* DNA or *M avium* subsp *silvaticum* DNA, for IS900- and IS902-PCR, respectively) was run in parallel with each series of polymerase chain reaction triplicates.

GEL ELECTROPHORESIS AND SOUTHERN BLOT HYBRIDISATION

Ten microlitre aliquots of the polymerase chain reaction mixtures were loaded onto 2% agarose gels for electrophoresis according to standard protocols, then Southern blotted onto nylon membranes (Hybond-N$^+$, Amersham, Bucks) in 0·4 M NaOH; 0·6 M NaCl. Membranes were hybridised at 65°C overnight with the 400 bp amplification product (pcr400) of IS900-PCR or the 252 bp amplification product (pcr252) of IS902-PCR, as appropriate. Radiolabelled pcr400 and pcr252 products were prepared according to the protocol of Feinberg and Vogelstein.[22] After hybridisation, membranes were washed at 65°C in 0·1×SSC; 0·1% SDS, as described previously.[13] Membranes were autoradiographed with intensifying screens at −70°C overnight.

Results

Recognisable acid fast bacilli were identified microscopically in 10 of the 31 long term cultures, originating from 29 patients' tissues. All 10 cultures were from mucosal biopsies; three cultures were from Crohn's disease tissues, four were from ulcerative colitis tissues, and three non-inflammatory bowel disease tissues. The remaining 21 cultures produced microscopically visible but uncharacterised spheroplasts.

The specificity of primers P102 and P103 for IS902 was demonstrated (Fig 1). DNA samples from the known isolates of *M avium* subsp *silvaticum* (lanes 7, 12, 23) were each IS902-PCR positive, showing the expected 252 bp amplification product. *M paratuberculosis*, containing the closely related IS900, all other *M avium* species and all other mycobacterial and enteric bacterial species tested were IS902-PCR negative. *Streptomyces ceolicolor* A3(2), which contains the related DNA insertion element IS110[17] was similarly IS902-PCR negative.

Results of the IS900- and IS902-PCR on the long term cultures are shown in the Table. *M paratuberculosis* positivity is established when IS900-PCR results in the amplification of a 400 bp product. Similarly, *M avium* subsp *silvaticum* specifically produces a 252 bp amplification product when submitted to IS902-PCR. Cultures were considered positive when the correct polymerase chain reaction amplification product was obtained in one, two, or all three

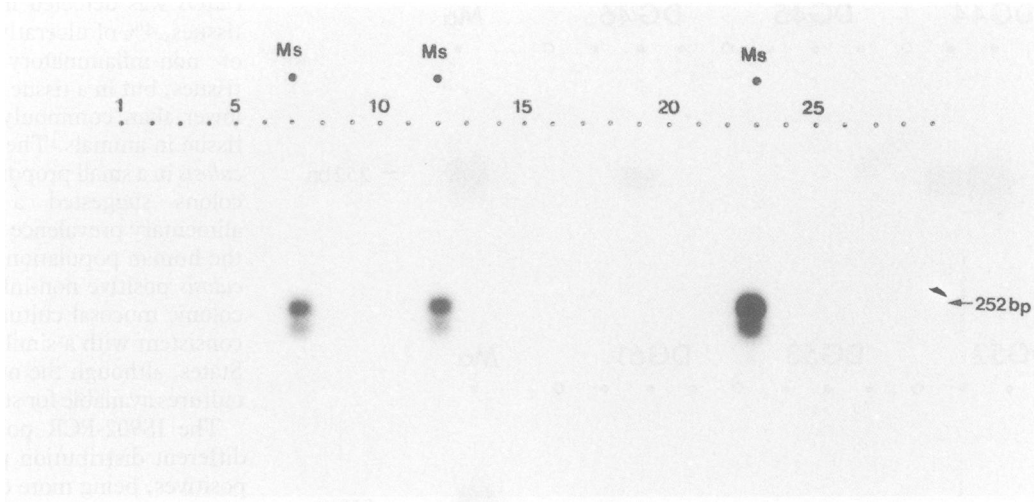


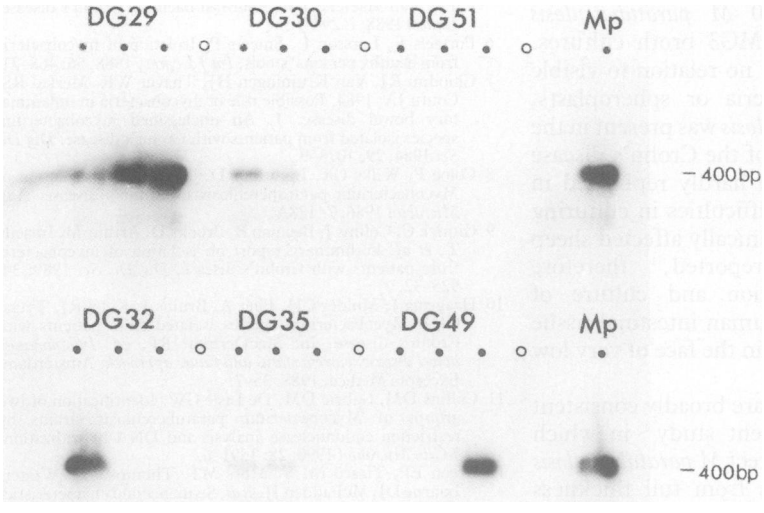
Figure 1: Autoradiograph showing the specificity of IS902 PCR for M avium *subsp* silvaticum *amplifying the predicted 252 bp product. A minor product of slightly lower molecular weight sometimes occurring in this reaction is also seen. Mycobacterial and other microbial DNA samples correspond to the following lanes: 1 and 2* – M avium *sp; 3 to 5* – M avium *AIDS isolates; 6* – M scrofulaceum; *7, 12, and 23* – M avium *subsp* silvaticum; *8* – M paratuberculosis; *9* – M tuberculosis; *10* – M africanum; *11* – M bovis; *13* – M kansasii; *14* – M fortuitum; *15* – M xenopi; *16* – M gordonae; *17* – M malmoense; *18* – M phlei; *19* – M marinum; *20* – M chelonei; *21* – M smegmatis; *22* – M szulgai; *24* – Anerobic peptococcus; *25* – Streptococcus faecalis; *26* – Escherichia coli; *27* – Streptomyces coelicolor; *28* – *buffer-only DNA extraction control; 29* – *no DNA template PCR control.*

lanes of the polymerase chain reaction triplicates, after high stringency washing of the Southern blots. Autoradiographs illustrating the results of IS900- and IS902-PCR on the long term cultures are shown in Figures 2 and 3.

Seven of the 31 cultures were positive for *M paratuberculosis*. Six of these positive cultures were from Crohn's disease patients, five of them originating from surgically resected tissue. A colonic mucosal biopsy obtained at endoscopic follow up from a non-inflammatory bowel disease patient with a colonic polyp was also positive for *M paratuberculosis*. Each of the six positive Crohn's disease cultures also grew spheroplasts. The positive non-inflammatory bowel disease culture also grew acid fast bacilli which were not *M paratuberculosis*. A further six cultures were positive for *M avium* subsp *silvaticum*, two each from the Crohn's disease, ulcerative colitis, and non-inflammatory bowel disease groups. The positive Crohn's disease cultures were both from resected tissue and also grew spheroplasts. Both ulcerative colitis cultures were of biopsy material and also grew acid fast bacilli. The two non-inflammatory bowel disease cultures were from mucosal biopsies, one grew spheroplasts and the other acid fast bacilli. Throughout the study the results of IS900 and IS902 polymerase chain reaction bore no relation to spheroplasts or bacillary form acid fast bacilli visible in the cultures.

The cryptic *M paratuberculosis* positive control culture (sample 36) was very strongly IS900-PCR positive and was also IS902-PCR negative. No false-positives or false-negatives were obtained with the MG3 medium only control or with the remaining three mycobacterial control cultures, with either polymerase chain reaction assay. None of the 62 buffer only polymerase chain reaction negative controls, run with each set of polymerase chain reaction triplicates, produced false-positives. All polymerase chain reaction positive controls (10 fg purified genomic DNA, theoretically equivalent to two bacilli) were positive.

Figure 2: Autoradiograph showing the detection of M paratuberculosis *DNA by IS900-PCR in five of six Crohn's disease cultures. DG29 shows triplicate positive signals. DG30 and DG35 show dulplicate positive signals. DG32 and DG49 show a single positive signal. DG51 was negative. 10 fg of* M paratuberculosis *DNA was amplified as a positive control (Mp). A no-DNA PCR negative control was submitted to PCR amplification with each set of triplicates (0).*



Discussion

In recent years there have been a number of studies reporting the isolation of mycobacteria from patients with Crohn's disease,[3-5] but it has been reports of the isolation of *M paratuberculosis*,[7-10] which have generated the greatest interest. This pathogenic organism is the causative agent of Johne's disease, a chronic ileitis/colitis of cattle and other animals, which shares many similarities with Crohn's disease.[23]

In the earlier studies, characterisation and identification of the mycobacterial isolates relied upon cultural and biochemical techniques. Polymerase chain reaction, however, is a powerful technique capable of amplifying specific target DNA sequences by a factor of 10^6.[24] Moreover, the sensitivity of polymerase chain reaction is increased when DNA sequences existing as multi copies within a genome, are targeted. Polymerase chain reaction amplification assays have been developed for the detection of a number of mycobacterial species[25-27] and have proven successful in the detection of mycobacteria from a variety of clinical specimens.[28] We have developed polymerase chain reaction assays for the detection of *M paratuberculosis* and *M avium* subsp *silvaticum*, based on their respective multi copy, species specific insertion elements, IS900 and IS902. The high specificity of insertion sequence based polymerase chain reaction tests, for the detection of *M paratuber-*

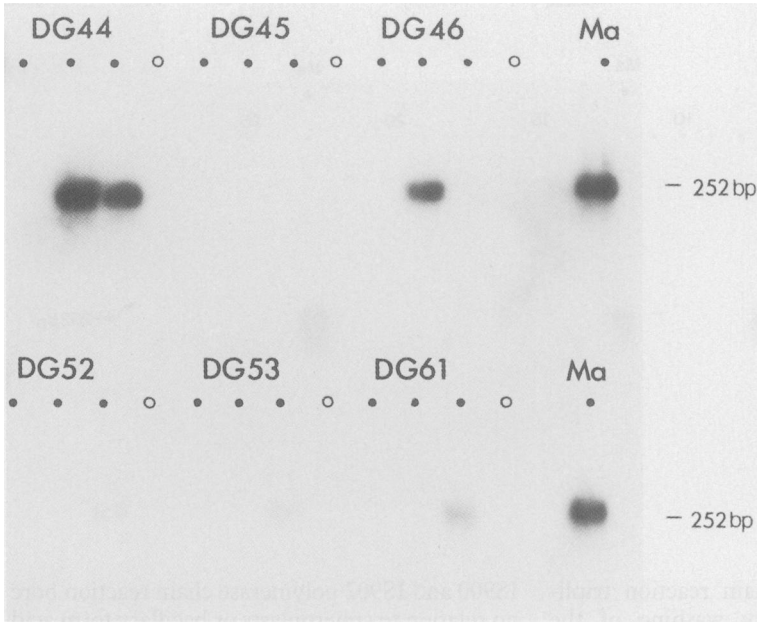


Figure 3: Autoradiograph showing the detection of M avium *subsp* silvaticum *DNA in long term cultures of intestinal tissue by IS902-PCR. DG44 (UC) shows duplicate positive signals. DG46 (non-IBD control), DG52 (CD), DG53 (non-IBD control), and DG61 (UC) show a single positive signal. DG45 (UC) was negative. 100 fg of* M avium *subsp* silvaticum *DNA was amplified as a positive control (Ma). A no-DNA PCR negative control was submitted to PCR amplification with each set of triplicates (0).*

culosis and *M avium* subsp *silvaticum* permits these known chronic enteric pathogens to be identified amongst a wide variety of other environmental mycobacteria which are normal inhabitants of the alimentary tract.[6] Growth of these normal mycobacteria in cultures from Crohn's disease, ulcerative colitis, and non-inflammatory bowel disease is to be expected.

In this present study undertaken blind, IS900-PCR detected *M paratuberculosis* in one third (six of 18) of long term broth cultures of intestinal tissue from Crohn's disease patients and in a single (one of six) non-inflammatory bowel disease control culture but in none of the ulcerative colitis cultures (nil of seven). For the six *M paratuberculosis* positive Crohn's disease cultures, positive amplification signals were observed in one of three or two of three lanes of the polymerase chain reaction triplicates. Moreover, the strength of the positive signals broadly equated to that obtained with the IS900-PCR positive control (equivalent to two *M paratuberculosis* genomes) and suggests that the assay was operating at or near the limit of its sensitivity. Thus the intensity and number of polymerase chain reaction positive signals indicates the presence of only 30–200 *M paratuberculosis* genomes in the original MG3 broth cultures. These results, which bore no relation to visible bacillary form mycobacteria or spheroplasts, suggest that *M paratuberculosis* was present in the original intestinal tissues of the Crohn's disease patients, in a form which hardly replicated in broth culture, if at all. Difficulties in culturing *M paratuberculosis* from clinically affected sheep have previously been reported,[11] therefore difficulties in the isolation and culture of *M paratuberculosis* from human intestinal tissue is perhaps to be expected, in the face of very low microbial abundance.

Our IS900-PCR results are broadly consistent with the results of a recent study[21] in which IS900-PCR was used to detect *M paratuberculosis* directly in DNA extracts from full thickness samples of human gut wall obtained from 103 people living in southern England. *M paratuberculosis* was detected in 65% of Crohn's disease tissues, 4% of ulcerative colitis tissues, and 12% of non-inflammatory bowel disease control tissues, but in a tissue abundance 10^5 to 10^7 fold lower than commonly seen in Johne's disease tissue in animals. The presence of *M paratuberculosis* in a small proportion of apparently normal colons suggested a previously unexpected alimentary prevalence of this animal pathogen in the human population. The single *M paratuberculosis* positive non-inflammatory bowel disease colonic mucosal culture in the present study is consistent with a similar situation in the United States, although the number of these long term cultures available for study was, of course, small.

The IS902-PCR positive cultures showed a different distribution profile to the IS900-PCR positives, being more equally spread among the Crohn's disease, ulcerative colitis, and non-inflammatory bowel disease control cultures. If the higher rate of detection of *M paratuberculosis* found in the Crohn's disease cultures were simply a consequence of the disease process itself, then it might reasonably be expected that the closely related *M avium* subsp *silvaticum* would be similarly distributed. The IS902-PCR results may suggest that *M avium* subsp *silvaticum* is less likely to be involved in the pathogenesis of Crohn's disease but may add to the significance of the IS900-PCR results.

M paratuberculosis is a slow growing intestinal pathogen, known to be able to cause chronic enteritis in a wide variety of animal species including cattle, goats, sheep, deer, horses and pigs,[1] rabbits,[29] and primates.[30] Further work is necessary to clarify the role of this environmental organism in chronic enteritis in man.

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